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K^+-driven sinusoidal efflux of glutathione disulfide under oxidative stress in the perfused rat liver

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Tert-butyl hydroperoxide (BHP), hydrogen peroxide and diamide caused a rapid and simultaneous release of glutathione disulfide (GSSG) and K^+ in the isolated perfused rat liver. Both BHP-induced effluxes were suppressed by prior depletion of hepatic glutathione, but not by co-infusion of desferrioxamine which prevented lipid peroxidation and cell death. High K^+ media decreased the GSSG efflux even though hepatic GSSG levels remained high. The GSSG and K^+ effluxes were repeatable if cellular K^+ recovered after a short BHP exposure. Ouabain inhibited the K^+ re-uptake and decreased the response to repeated BHP challenge. Thus, sinusoidal efflux of GSSG under oxidative stress may be driven by a K^+ gradient.

Glutathione disulfide efflux; K^+ efflux; Oxidative stress; Isolated perfused rat liver

1. INTRODUCTION

The efflux of glutathione disulfide (GSSG) under oxidative stress has been previously reported in various biological systems, such as eye lens [1], erythrocytes [2], perfused liver [3–9], isolated hepatocytes [10], and perfused heart [11,12], and is considered as a reliable index of cellular oxidative stress. The liver has two GSSG transport systems of which excretion into the bile through the canalicular membranes, in which ATP-driven and carrier-mediated mechanisms have been proposed [10,13–15], is primary. The other is sinusoidal release which is thought to ensue in response to excess cellular accumulation of GSSG when the canalicular transport system is saturated [3–8]. Using isolated perfused liver, Sies et al. [3] first demonstrated that GSSG was released into the effluent perfusate when cellular GSH was oxidized by infusing high concentrations of the glutathione peroxidase substrates, tert-butyl hydroperoxide (BHT), cumene hydroperoxide and hydrogen peroxide. This sinusoidal GSSG release with toxic insult is an early event with a net loss of cellular glutathione, and therefore may have a significant role in the subsequent development of toxicity. However, the mechanism of the release is not understood. The present study using perfused liver suggests that sinusoidal GSSG efflux under oxidative stress is driven by the K^+ concentration gradient across the plasma membrane accompanying the efflux of K^+.

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2. MATERIALS AND METHODS

2.1. *Liver perfusion*

The livers were isolated from male Sprague–Dawley rats (170–180 g, given food and water ad libitum) between 9.30 and 12.00 h according to the basic procedure except that the smaller lobes (proc. papillaris, proc. caudatus and lobus dexter) were tied and removed [16]. The bile duct was cut close to the liver and the bile was free to drain. Perfusion was performed with a non-recirculating and constant flow (25 ml/min) system using Krebs Henseleit bicarbonate buffer (KHB; 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 5.6 mM glucose, saturated with 95% O_2/5% CO_2 at 37°C). Modification of the ionic composition was described in the legends. Experiments commenced exactly 30 min after cannulation of the portal vein. BHP, hydrogen peroxide, diamide, desferrioxamine and ouabain were dissolved in KHB and infused at a rate less than 0.25 ml/min using an infusion pump (Neuroscience Inc.).

2.2. *Effluent monitoring*

The outlet of the perfusion system was connected to a thermister, an oxygen electrode (Clark type) and a K^+ ion electrode (Orion). The effluent perfusate was collected for 15 min at appropriate intervals. Thiobarbituric acid-reactive substances (TBARS) and lactic dehydrogenase (LDH) activity in the perfusate, as indices of lipid peroxidation and cell death, were determined as described previously [16]. TBARS values were expressed as malondialdehyde equivalents. GSSG concentration in the perfusate was assayed by following the oxidation of NADPH at 340 nm after addition of glutathione reductase [5].

2.3. *Hepatic glutathione assay*

The perfused liver was instantaneously freeze-clamped under liquid nitrogen, deproteinized by 5 vol. of 5% sulfosalicylic acid, and the total glutathione (GSH + GSSG) and GSSG in the acid extract were determined by the method of Tietze [17] and Griffith [18].

3. RESULTS AND DISCUSSION

Although Sies et al. [4] originally reported that K^+ was released into the effluent perfusate by infusing BHT

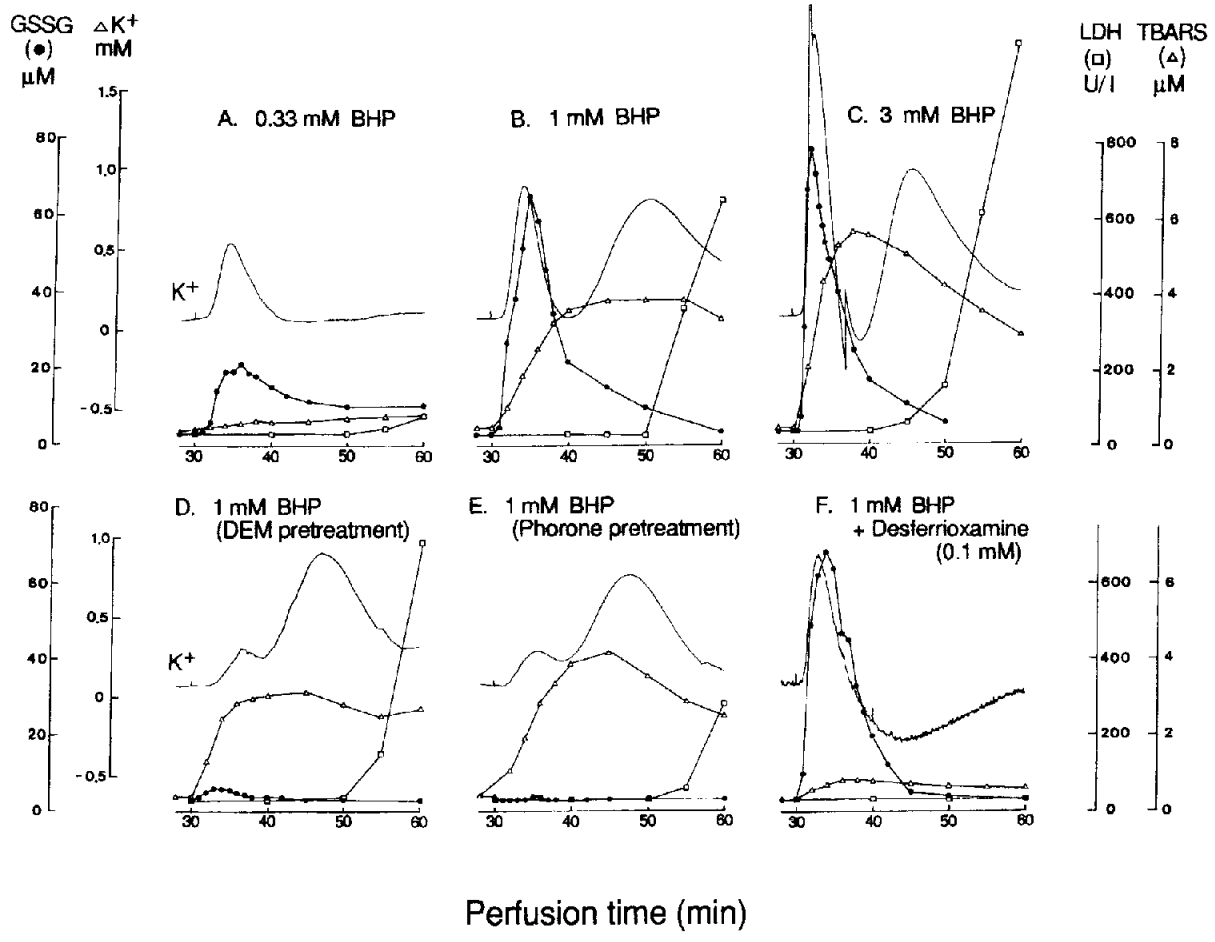

Fig. 1. Release of GSSG, K^+, TBARS and LDH into the effluent perfusate by BHP infusion in perfused rat liver under various conditions. Infusion of BHP was begun at 30 min after cannulation. In D and E, rats were pretreated with diethylmaleate (DEM, 1 ml/kg, i.p.) and phorone (250 mg/kg, i.p.) 2 h before liver isolation. In F, infusion of 0.1 mM desferrioxamine was begun 10 min before BHP infusion. Traces show changes of K^+ concentration. TBARS values are expressed as malondialdehyde equivalents. Representatives of each 2–4 experiments are shown.

into the perfused rat liver, the relationship between the K^+ release and GSSG efflux has not been determined.

As traces in Fig. 1A, B and C show, continuous infusion of 0.33 to 3 mM BHP caused a biphasic release of K^+, although the later release was minimal with 0.33 mM BHP. This K^+ release was compensated by Na^+ uptake (data not shown). The initial rapid K^+ release occurred concominantly with a release of GSSG depending on the concentration of BHP, the greater the initial GSSG release the shorter the duration of the release. The latter slower and greater K^+ release at 1 and 3 mM BHP, just preceding LDH leakage, may be related to the subsequent cell death. In agreement with previous reports [5,9,19], a rapid and sustained release of TBARS occurred.

The simultaneous release of GSSG and K^+ following 1 mM BHP infusion was markedly suppressed in the glutathione-depleted livers isolated from animals pretreated with diethylmaleate or phorone (Figs. 1D and E), but leakage of TBARS and LDH as well as the latter K^+ release occurred. Conversely, coinfusion of desferrioxamine, an iron chelater which is known to prevent lipid peroxidation [9,20], almost completely prevented TBARS and LDH release as well as the latter K^+ leakage, but did not affect the initial release of K^+ and GSSG (Fig. 1E). These findings indicate that the early release of GSSG and K^+ in itself is a phenomenon that is independent of cell damage, and that the suspected plasma membrane permeability change due to enhanced lipid peroxidation may not be the primary cause of GSSG release. In addition, the concomitant release of GSSG and K^+ appears universal since it was also observed with the other glutathione oxidants, hydrogen peroxide and diamide (Fig. 2), and with preliminary experiments using menadione (data not shown).

Therefore, in an attempt to determine whether GSSG efflux was mechanistically coupled with K^+ efflux, we examined the effect of high extracellular K^+ concentrations on GSSG release. As shown in Fig. 3, GSSG release due to 5-min infusion of 1 mM BHP decreased with increasing KCl concentration of the perfusate from 4.8 mM (regular KHB) to 118 mM. In this experiment,

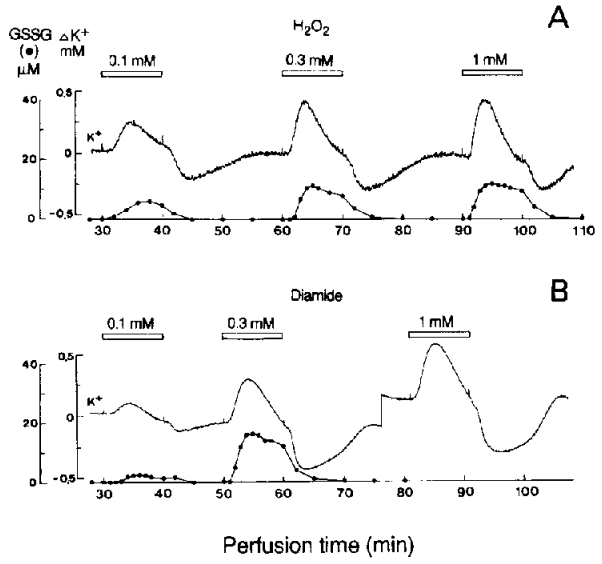

Fig. 2. Simultaneous release of GSSG and K^+ by infusion of hydrogen peroxide and diamide in perfused rat liver. Infusion of 1 mM diamide disturbed determination of the effluent GSSG concentration. No LDH leakage was observed during the experimental periods in either A or B.

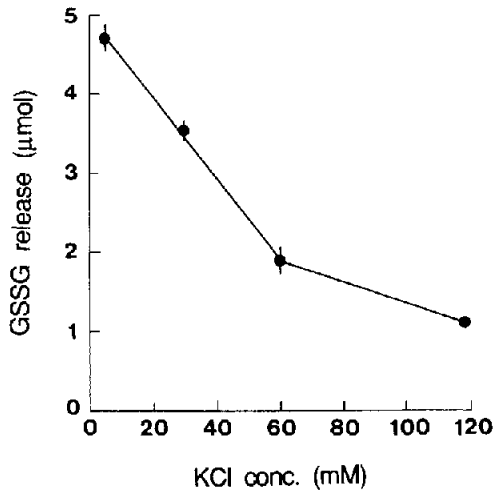

Fig. 3. Effect of perfusate KCl concentration on BHP-induced GSSG release in perfused rat liver. The KCl/NaCl concentration ratio (in mM) of the perfusate was changed as follows: 4.8/112 (regular KHB buffer), 30/87, 60/57 and 112/4.8. The perfusion medium was changed to high K^+ medium at 20 min, 1 mM BHP was infused at 30 min for 5 min, and the effluent perfusate collected every 1 min. Total GSSG released into the effluent during the initial 10 min was calculated. Each point represents the mean of two experiments with the range.

when high K^+ medium was returned to regular KHB after the first BHP challenge, hepatic GSSG release well responded to the second BHP challenge (data not shown), indicating that the K^+ effect is reversible.

The K^+ effect was further confirmed by comparing glutathione content of liver perfused with regular and high K^+ (118 mM) medium and infused with 1 mM BHP (Table I). Since perfusion with high K^+ medium caused a considerable swelling of the liver (the liver weight/body weight ratios at the end of perfusion in regular KHB and high K^+ medium groups were 3.2 ± 0.2 and 4.5 ± 0.2% (mean ± S.D., n = 4–5), respectively, with no significant alteration with BHP infusion), glutathione content per liver (values in the parentheses in Table I), rather than per gram liver, may reflect the net change between both groups. Thus, in the control liver, total glutathione content was not significantly affected by high K^+ medium. Infusion of BHP for 10 min in the regular KHB group decreased the total glutathione content to about half the control accompanying a marked

Table I

Effect of BHP on hepatic glutathione content in livers perfused with regular KHB and high K^+ medium

		Total [GSH + GSSG] (μmol GSH equivalent)	GSSG (μmol GSH equivalent)	GSSG/total (%)
Regular KHB	Control	7.80 ± 0.77 #	0.084 ± 0.012	1.1 ± 0.2
	n = 5	(42.3 ± 7.2)##	(0.48 ± 0.11)	
Regular KHB	1 mM BHP	3.96 ± 0.43	2.23 ± 0.38	56.8 ± 11.5
	n = 4	(20.8 ± 2.5)	(11.8 ± 2.3)	
High K^+ medium	Control	5.40 ± 0.32	0.070 ± 0.014	1.3 ± 0.3
	n = 4	(40.1 ± 2.7)	(0.53 ± 0.13)	
High K^+ medium	1 mM BHP	4.05 ± 0.31	2.08 ± 0.32	51.5 ± 5.3
	n = 4	(32.3 ± 2.4)*	(16.6 ± 2.4)**	

Regular KHB group: infusion of 1 mM BHP was begun at 30 min. High K^+ group: the perfusate was exchanged from regular KHB to the high K^+ medium containing 118 mM KCl and 4.8 mM NaCl (See legend of Fig. 3) 10 min before BHP infusion. In both groups, after 10 min of BHP infusion the liver was immediately freeze-clamped and the glutathione content measured as described in the materials and methods section.

#Per g liver; ##Per whole perfused liver. Values are the mean ± S.D. *-**Significantly higher than the regular KHB/1 mM BHP group at *$P < 0.01$ and **$P < 0.05$.

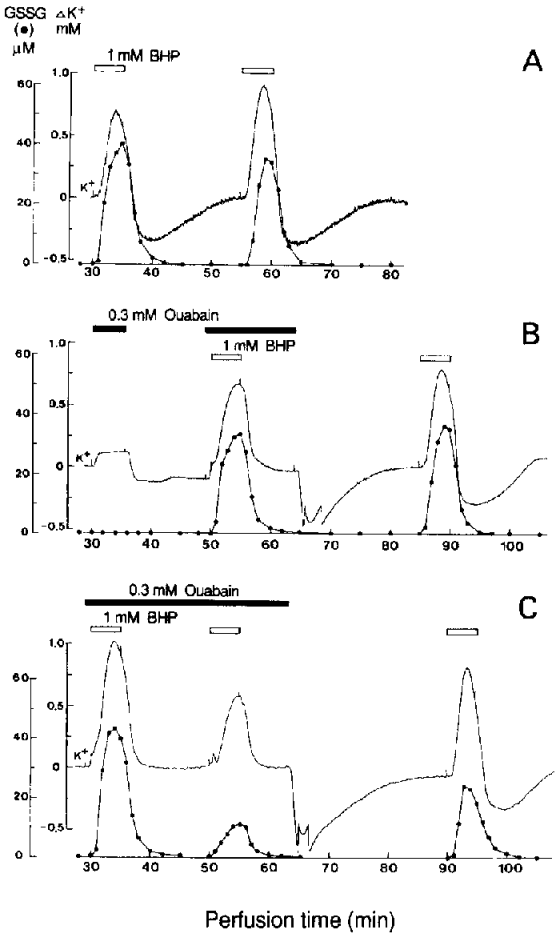

Fig. 4. Effects of ouabain on the release of GSSG and K^+ induced by short-term infusion of BHP in perfused rat liver. (A) Control, repeated short-term (5 min) infusion of 1 mM BHP. (B) Single BHP infusion in the presence of ouabain. (C) Repeated BHP infusion in the presence of ouabain.

increase in GSSG. In the high K^+ group, the total glutathione and GSSG contents after BHP infusion remained significantly higher than those in the regular KHB group. The GSSG/total glutathione ratio reached more than 50% with no significant difference between the groups. These observations indicate that under high extracellular K^+ the intracellular GSH oxidation system is still active but the accumulated GSSG cannot be released probably due to a decrease in K^+ gradient. Thus, GSSG efflux seems to be mechanistically coupled with K^+ efflux, for which a K^+ gradient across the plasma membrane is critical. In this experiment, it should also be noted that the hepatic glutathione content was measured 10 min after infusion of 1 mM BHP, when the release of GSSG and K^+ into the effluent had almost ceased (Fig. 1B). The rapid decline of GSSG and K^+ concentrations in the effluent perfusate therefore may not be due to a decreased intracellular GSSG content but rather due to a cellular loss of K^+, which was confirmed by the following experiments.

Although the GSSG release was transient under continued infusion of BHP, it was repeatable if infusion of BHP (1 mM) was transient (5 min) (Fig. 4A). Under such conditions, K^+ release was soon followed by its re-uptake. This K^+ re-uptake phase was inhibited by ouabain, but immediately appeared after its removal (Fig. 4B), indicating that the released K^+ is taken up by the Na^+-K^+ pump. Furthermore, under conditions of inhibited K^+ re-uptake, the BHP-induced release of K^+ and GSSG decreased (Fig. 4C). These observations also indicate that intracellular K^+ concentration has a crucial role in GSSG release.

Thus, efflux of GSSG from hepatocytes may utilize the energy of the K^+ efflux down the concentration gradient. According to tentative calculations of the amounts of GSSG and K^+ released with 5-min infusion of 1 mM BHP (the first challenge) in the presence of ouabain, i.e. under cessation of K^+ re-uptake, about 20 equivalents (18.8 ± 1.7, $n = 4$) of K^+ may be transported per mole of GSSG. Thus, hepatocytes seem to dispose of the excess physiological oxidant GSSG at the expense of considerable loss of cellular K^+. Considering that the action of BHP under the present experimental conditions is localized to the periportal zones of the liver lobules [9] where glutathione peroxidase is also concentrated [21], such a loss of K^+ may aggravate succeeding oxidant-specific cell damage as far as lack of recovery of K^+ loss.

We consider that the efflux of GSSG and K^+ occurs largely via sinusoidal plasma membranes of hepatocytes rather than via the paracellular mechanism, i.e. efflux from bile to perfusate through junctional complex [15], since ligation of the bile duct had no significant effect on the extent of the release of GSSG and K^+ induced by infusion of 0.33 and 1.0 mM BHP (data not shown). However, a minor contribution of the latter mechanism cannot be ruled out.

The canalicular transport of GSSG into bile, like glutathione conjugate, is considered to be mediated by an active ATP-dependent process using the same carrier system [10,13,14]. Under oxidative stress, an ATP-dependent transport system has also been proposed for GSSG efflux from erythrocytes [2,22] and heart [12], in which tissues no particular excretion devices are histologically identifiable on the plasma membranes. GSH efflux from hepatocytes is reportedly an electrochemical process dependent on the K^+ gradient [23]. The present study suggests that the hepatic sinusoidal release of GSSG under oxidative stress is also driven and regulated by the K^+ gradient. Whether this process is ATP-dependent is still unknown, but ATP is required indirectly to recover the loss of cellular K^+ by means of the Na^+-K^+ pump.

The mechanism of the cotransport remains to be elucidated. The increased GSSG/GSH ratio could modify plasma membrane proteins by disulfide or mixed sulfide formation to cause changes in membrane permeability.

In *E. coli*, glutathione-regulated K^+ efflux systems known as KefB and KefC, which are activated by glutathione metabolites, have been identified [24,25]. Involvement of such a specific transport system in hepatocyte plasma membranes is another interesting possibility.

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